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BRIEF REPORT

Molecular characterization of a foot-and-mouth disease virus containing a 57-nucleotide insertion in the 3′untranslated region

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Abstract A foot-and-mouth disease virus containing a 57-nucleotide (nt) insertion in the 3′untranslated region (3′UTR) was generated by transposon (tn)-mediated mutagenesis. Characterization of the mutant virus (A24-3′UTR8110) revealed no significant differences in virus growth, translation efficiency or virulence in cattle compared to the A24 wild-type virus. RNA modeling showed that the structures predicted in the 3′UTR were not affected by the tn insertion. These results revealed that the 3′UTR can tolerate foreign sequences that do not disrupt essential signals required for virus replication.

Foot-and-mouth disease virus (FMDV), an aphthovirus, belongs to the family *Picornaviridae* along with the entero-, rhino- and cardioviruses. Picornaviruses are small, positive-strand RNA viruses. The FMDV genome is approximately 8,300 nt in length and contains a single large open reading frame (ORF) that is flanked by 5′ and 3′untranslated regions (UTRs). The ORF is translated into a long polyprotein, which is proteolytically processed by viral proteases to produce the mature viral proteins [5]. The 5′ and 3′UTRs have important elements (primary sequence or secondary structures) required for viral replication [7, 8]. The 5′UTR is about 1,200 nt long and contains an S fragment, a poly(C) tract, several pseudoknots, a stem-loop cis-acting replication element (CRE) and a highly structured internal ribosome entry site (IRES), while the 3′UTR is about 90 nt long and contains a poly(A) tail. The 3′UTR of FMDV is composed of two stem-loops linked by a variable region, and nucleotide sequence analysis has revealed that it is less conserved that the 5′UTR [3].

The first step in FMDV replication is translation of the viral RNA genome into a single polyprotein by a cap-independent mechanism controlled by the 5′UTR [2]. Cleavage of the polyprotein leads to the production of the viral structural and nonstructural proteins. Synthesis of the viral minus- and plus-strand RNA takes place in replication complexes by poorly understood mechanisms and requires viral and cellular proteins [1]. Serrano et al. [17] have recently demonstrated a direct base-pairing interaction between the 5′UTR and the 3′UTR and identified cellular proteins that bind to both UTRs. These interactions support the potential circularization of the viral genome and may regulate the balance between viral translation and replication. The role of the 3′UTR in viral replication has been examined by substitution of the FMDV 3′UTR with that of another picornavirus and by its deletion in a full-length cDNA clone. In both cases, no virus was recovered, suggesting that this region is strictly required for viral replication and infectivity [16].

We have recently constructed a library of FMDV genomes (tn-FMDV) containing insertions at random sites, using a transposon-mediated mutagenesis approach [13].

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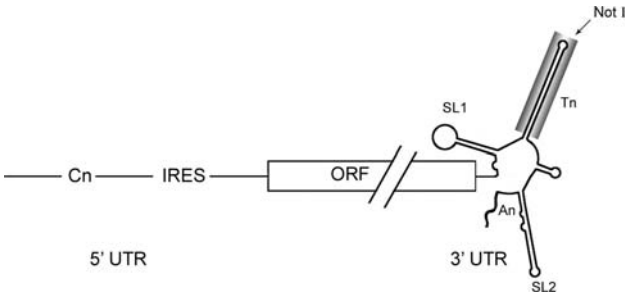

Fig. 1 Location of the transposon (tn) insertion in the genome of mutant virus A24–3′UTR8110. The *open box* indicates the viral open reading frame (ORF). Predicted RNA secondary structures within the 3′UTR are indicated: *SL1* stem loop 1; *SL2* stem loop 2; *tn* transposon (*shaded box*). The position of a unique NotI restriction site contained in the tn is shown. The number in the mutant name refers to the nt at which insertion occurred according to the FMDV A24 published sequence (GenBank accession number AY593768). *IRES* internal ribosome entry site; *UTR* untranslated region; *Cn* poly(C) tract; *An* poly(A) tract

Briefly, we have used the Ez-Tn In-Frame Linker Insertion kit (Epicentre Biotechnologies) to introduce 57-nt in-frame insertions into a plasmid encoding the complete FMDV A24 Cruzeiro genome (pA24). The location of each of the transposon (tn) insertions was mapped by sequencing, and mutant viruses were generated by RNA transcription and transfection. Remarkably, we were able to obtain a viable mutant virus containing a tn insertion in the highly structured 3′UTR. In this study, we have characterized this FMDV mutant, designated A24-3′UTR8110, which contains an insertion at position 8110. The effect of the insertion on virus growth, translation and virulence in cattle was evaluated.

Initially, BHK-21 cells were transfected by electroporation with equal amounts of RNA derived from parental pA24 and mutant pA24-3′UTR8110 plasmids. Cells transfected with parental RNA displayed cytopathic effects (CPE) within 16 h, whereas cells transfected with mutant RNA required an additional cell passage to develop CPE, indicating a delay compared to WT virus. Recovered viruses were passed three times in BHK-21 cells to generate viral stocks for subsequent studies. The presence of the tn insertion was confirmed by sequencing of this virus stock. Moreover, sequencing of the entire genome of the mutant virus revealed synonymous changes G3138A in the VP3 coding region and A4731C in the 2C coding region compared to the sequence of WT parental DNA. Figure 1 shows the position of the tn insertion in the FMDV genome.

First, we examined the plaque morphology of the A24-3′UTR8110 virus in bovine (LFBK) and porcine (IBRS-2) cell lines as well as secondary lamb kidney (LK) and

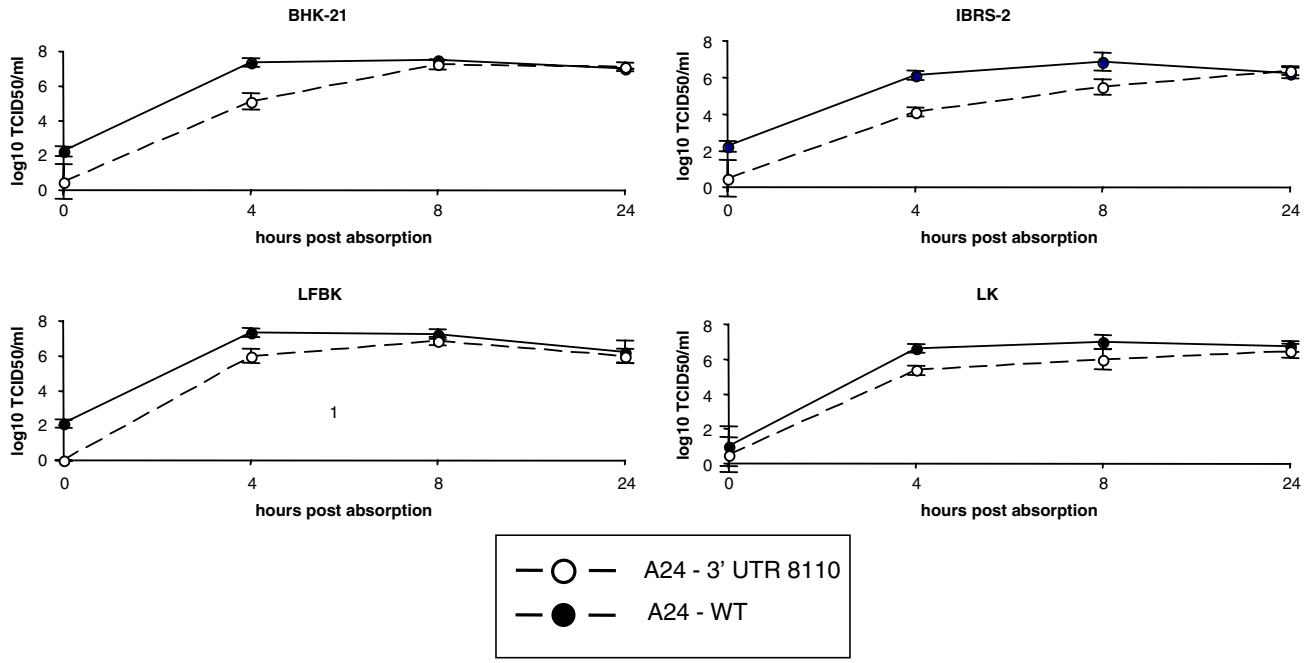

Fig. 2 One-step growth curve of A24–3′UTR8110 and WT viruses on different cell lines. Cell monolayers were infected for 1 h at 37°C at an MOI of 10, rinsed with 25 mM morpholineethanesulfonic acid (MES) buffer/145 mM NaCl, pH 6.0 (to eliminate residual input virus) and then incubated at 37°C. At the times indicated, cells and supernatant were frozen, and the virus released into the supernatants was titrated by TCID50 on BHK-21 cells. *Error bars* indicate values obtained from four different experiments

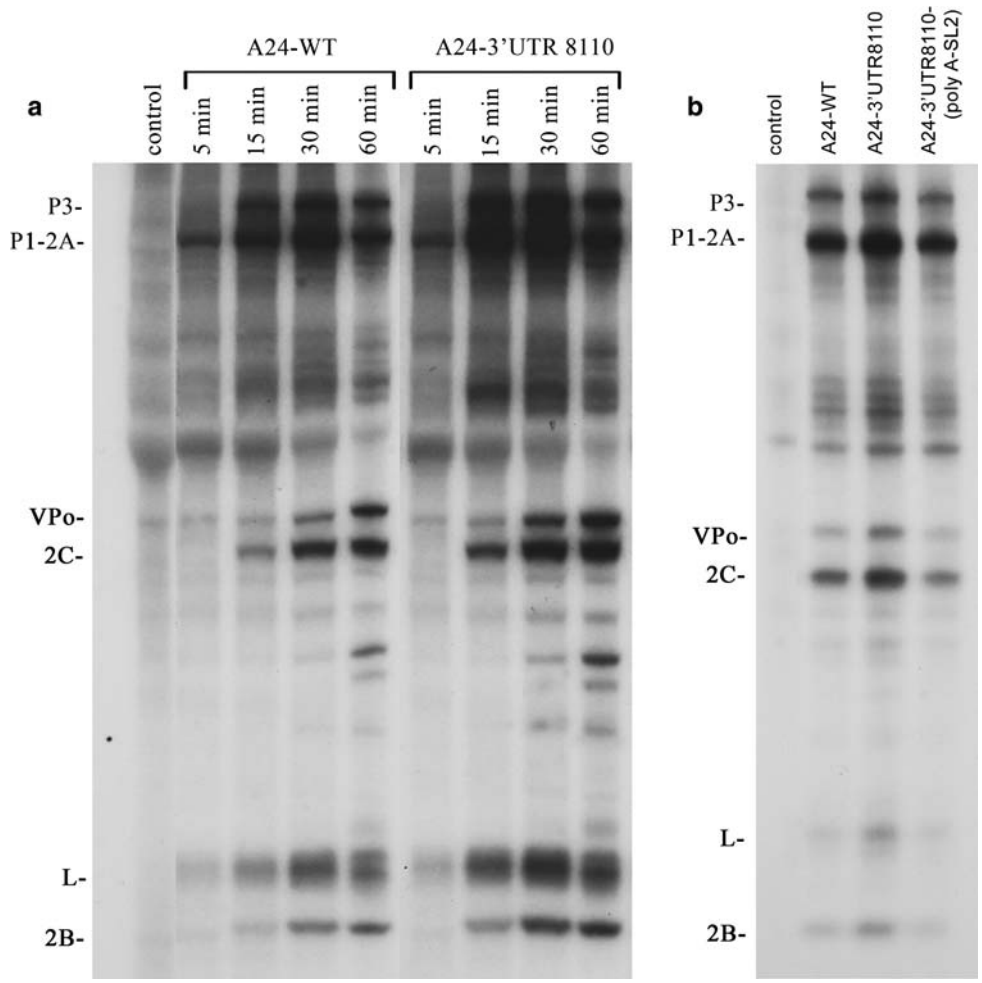

Fig. 3 Effect of the tn insertion on viral translation and processing in a cell-free in vitro translation system. The quality and quantity of the RNAs used in these experiments were determined in a nanodrop spectrophotometer. **a** IBRS-2 cell-free extracts were incubated with 400 ng of transcript RNAs derived from full-length WT pA24 or mutant pA24-3′UTR8110 plasmids in the presence [^{35}S] methionine for 1 h, and aliquots were removed at various times and analyzed on an SDS 12% polyacrylamide gel. The positions of viral proteins and precursors are indicated on the *left*. **b** Effect of 3′end sequences on the in vitro translation of the 3′UTR8110 RNA. 100 ng of transcript RNAs derived from WT pA24, mutant pA24-3′UTR8110 or NotI-digested mutant pA24-3′UTR8110 (lacking the SL2 and poly(A) tail) plasmids were used to program in vitro translation reactions in IBRS-2 cell extracts. The experimental procedure was as described in **a**, except that samples were taken after 1 h incubation

BHK-21 cells. In all cases, the A24-3′UTR8110 virus produced plaques about 2 mm smaller than those produced by WT virus (data not shown). We next analyzed the growth properties of A24-3′UTR8110 mutant virus by infecting a variety of cell lines at both low and high multiplicities of infection (MOIs) and determining the yield of infectious virus at various times after infection. Figure 2 shows growth curves of WT and mutant viruses in BHK-21, LFBK, IBRS-2 and LK cells at an MOI of 10. The A24-3′UTR8110 mutant virus replicated surprisingly well in all cells tested. Compared to WT virus, the mutant grew at a slightly slower rate, but by 24 h postinfection (hpi) both viruses reached similar titers, indicating that the cellular factor(s) required for virus infection interacted with the mutated 3′UTR in all cell types. Similar results were obtained at MOI 0.1 (data not shown).

Next, we investigated the effect of the tn insertion on viral RNA translation using cell-based in vitro translation systems [11, 14, 18]. IBRS-2 cell-free extracts were incubated with 400 ng of WT or mutant 3′UTR8110 RNA in the presence of [^{35}S] methionine. Samples were harvested at various times and analyzed by SDS-PAGE. Figure 3a shows that neither the kinetics of synthesis, the processing pattern of the viral proteins nor the intensity of each product varied substantially when WT or 3′UTR8110 RNA was translated. Similar results were obtained using a BHK-21 cell-free extract (data not shown).

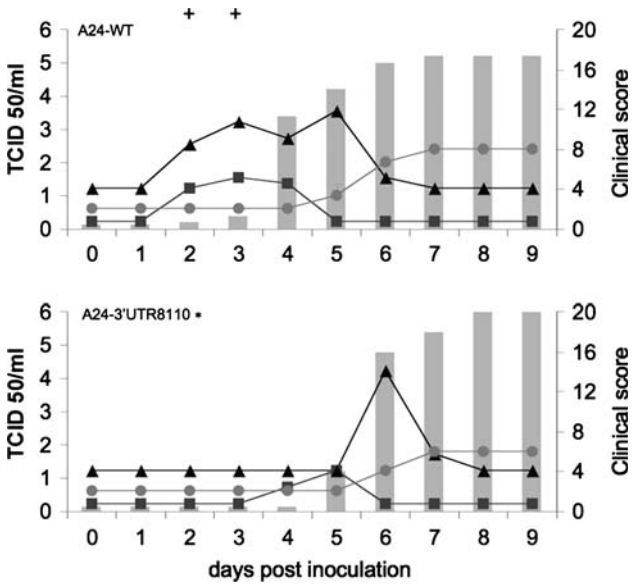

Fig. 4 Clinical and serological findings after aerosol inoculation of cattle with A24-WT or mutant A24-3′UTR8110 viruses. Cattle were inoculated with 10^7 TCID50 of each virus and monitored for 9 days after infection. Serum and saliva were collected daily and tested for the presence of virus and/or neutralizing antibodies against FMDV. Data for WT virus represents the average of three animals. *Plus* denotes fever (defined as a body temperature of >40°C) on days detected. Serum neutralizing antibody titers (*filled circles*), virus titers in blood (*filled squares*) and in saliva (*filled triangles*) are indicated on the *left axes*. Clinical scores (*shaded bars*) based on the presence of vesicle(s) in the four feet and mouth/tongue are shown on *right axes*. *Virus recovered from this animal retains the tn sequence

As shown in Fig. 1, the tn sequence introduced a unique NotI restriction site into the pA24-3′UTR8110 cDNA clone. By using this NotI site, we were able to remove the stem-loop 2 (SL2) and poly(A) tail region from the viral genome. When RNA (100 ng) derived from the NotI-digested mutant pA24-3′UTR8110 cDNA clone was incubated in IBRS-2 cell-free extracts, no apparent decrease in translation efficiency was observed compared to complete 3′UTR8110 or WT RNA (Fig. 3b). Similar data were obtained using a BHK-21 cell extract. In contrast to our results, Lopez de Quinto et al. [6] reported that these regions have a relevant function in viral translation in a rabbit-reticulocyte-lysate-based system. The discrepancy between our results and those of Lopez de Quinto et al. could be due to different translation factors present in the in vitro translation systems used in each study. In addition, RNA derived from NotI-digested pA24-3′UTR8110 was introduced by electroporation into BHK-21 cells, but no virus was recovered. Therefore, the deletion of the SL2 and/or the poly(A) tail in the 3′UTR did not significantly affect viral translation in cell-free synthesis systems but is critical for virus replication.

Finally, we analyzed the ability of the mutant virus to cause foot-and-mouth disease (FMD) in animals. Cattle were infected by aerosol with 1×10^7 TCID50/ml of mutant A24-3′UTR8110 or WT virus according to the model described by Pacheco et al. [12], and the results are shown in Fig. 4. The cow infected with mutant A24-3′UTR8110 virus exhibited a delay in the appearance of clinical signs of FMD compared to cattle infected with WT virus. This cow showed typical lesions of FMD after 5–6 days postinfection (dpi), but no fever was detected at any time after infection. The presence of virus in saliva and blood of this cow was also delayed compared to cattle infected with WT virus. Analyses of serum samples showed that all animals developed neutralizing antibodies, confirming infection with FMDV. Viruses recovered from tongue epithelium and vesicular fluid at 6 dpi in the cow infected with mutant A24-3′UTR8110 were sequenced in the region containing the tn insertion. We found that all viruses contained the transposon, and, interestingly, all contained the same nt change (A → G) within the tn sequence itself (data not shown). However, we could not exclude the possibility of additional mutation(s) outside the sequenced 3′UTR of the recovered viruses [4]. These results indicated that the tn insertion had little effect on virulence in cattle.

To evaluate the impact of the tn insertion on the secondary structure of the 3′UTR, we analyzed the 3′end of the mutant genome using the mfold program [20]. The predicted secondary structure for the 3′UTR of A24-3′UTR8110 showed that the tn folds to form an extra stem-loop located in the region connecting the two conserved SL structures (Fig. 5). To investigate whether the secondary structure of the mutant A24-3UTR8110 resembles that of any natural isolate, we analyzed the 3′UTRs of all FMDVs for which sequences were available. All viruses exhibited two similar SL structures among their potential secondary structures of minimum energy. On the other hand, the tn insertion appeared not to disturb the proposed loop-loop "kissing" interaction, as has been suggested for other picornaviruses [9, 10, 19].

Rodriguez-Pulido et al. [15] have recently reported that deletion of a 30-nt region, predicted to form the SL1 in the 3′UTR on an FMDV type O infectious clone, resulted in viruses with slower growth kinetics in cell culture and induced attenuation in swine. Our results show that we were not able to recover viruses lacking the SL2 and poly(A) tail. Since these structures are critical for virus infectivity/viability, we concluded that the tn insertion in the A24-3′UTR8110 virus preserved the general format of the 3′UTR structure. A detailed mutational analysis of the 3′UTR is needed to elucidate

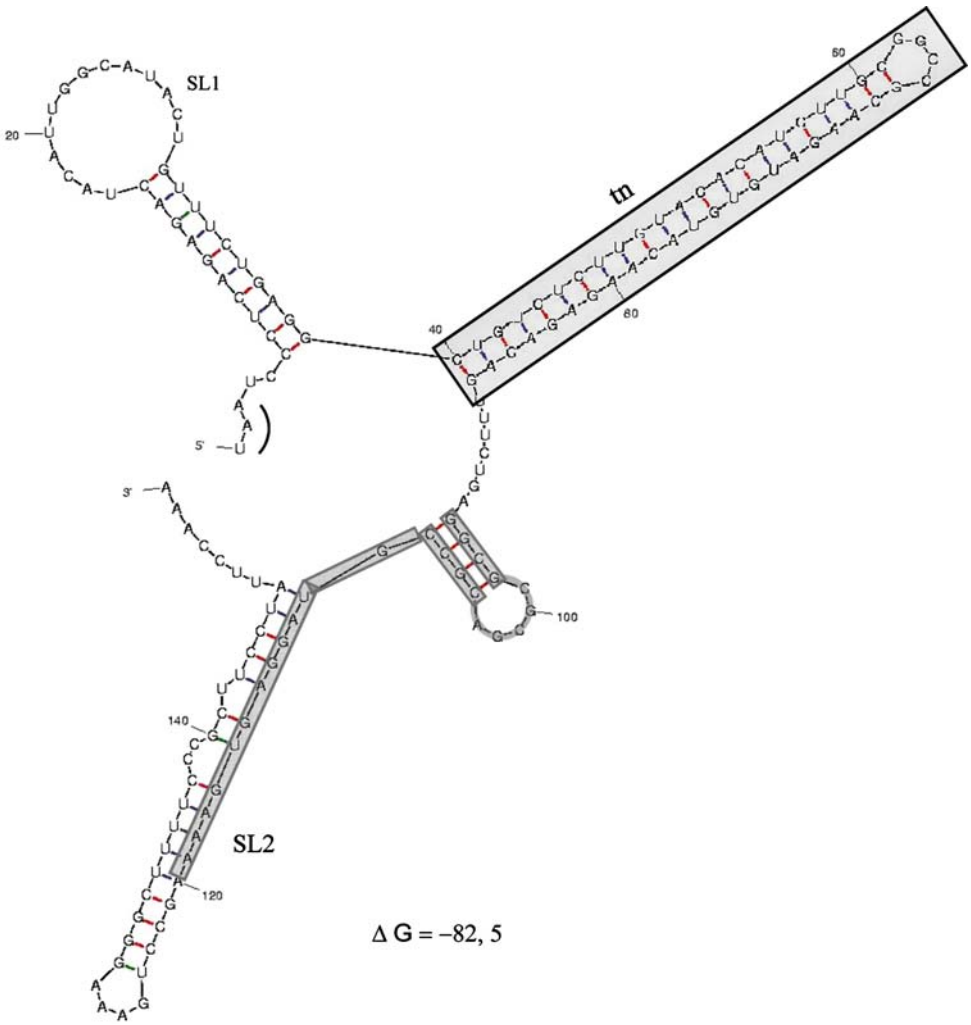

Fig. 5 Structure of the 3′UTR of the A24-3′UTR8110 virus. The RNA secondary structure of the mutant virus 3′UTR was obtained using the mfold program [20]. The stop codon is *underlined*, and the tn insertion is *boxed*. The RNA sequence is numbered from the stop codon, i.e. the first U residue is number 1. *Gray shading* indicates a highly conserved motif described by Carrillo et al. [3]

the biological function of these RNA structures in virus replication.

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